Growth requirements *in vitro* of oligodendrocyte cell lines and neonatal rat brain oligodendrocytes

(CO-13-7 cell line/insulin/transferrin/fibronectin/polylysine)

JANE E. BOTTENSTEIN

Marine Biomedical Institute and Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, TX 77550-2772

Communicated by Gordon H. Sato, November 15, 1985

ABSTRACT I have defined the basic requirements for the proliferation of cell lines expressing oligodendrocyte properties and for the survival of galactocerebroside-positive oligodendrocytes derived from neonatal rat brains. Conventional serum-containing medium can be replaced by O1 medium, a chemically defined medium supplemented with insulin, transferrin, sodium selenite, and biotin. Thyroid hormone is not required. When cells are plated directly into O1 medium, the substratum has to be modified by precoating with polylysine and adding fibronectin to the medium prior to the cells. Both cell lines and brain cells can be subcultured numerous times in O1 medium without initial culture in serum-containing medium. Brain cultures can be maintained in O1 medium for several months and contain a significantly higher percentage of mature oligodendrocytes, a lower number of astrocytes, and no fibroblasts as compared to cells maintained in serum-containing medium.

Oligodendrocytes in the central nervous system (CNS) and Schwann cells in the peripheral nervous system are responsible for forming multilayered myelin sheaths around neuronal axons, which increases their conduction velocity and consequently the efficiency of information transfer. It has been reported that some cultured oligodendrocytes are directly depolarized by γ -aminobutyric acid (1) and exhibit diaminobutyric acid-sensitive γ -aminobutyric acid uptake (2), suggesting an additional role in modulating neuronal function. Although vertebrate cell culture studies have yielded important information about Schwann cell division, maturation, and interaction with neurons, much less is known about oligodendrocytes. Clearly, an understanding of the molecular regulation of survival, growth, and maturation of oligodendrocytes is important for the analysis of developmental and pathological processes.

Oligodendrocytes from late-term embryonic, neonatal, and adult CNS of several mammals have been maintained *in vitro* by using conventional culture methods (3). These consist of a synthetic culture medium supplemented with undefined biological fluids, such as serum, plasma, and/or embryo extract, and culture surfaces unmodified or coated with collagen or polylysine. Most data reported describe dissociated cultures of mixed cellular phenotypes, although there are reports of enrichment for oligodendrocytes (3). In neonatal CNS cultures, although neurons are present in the initial population of cells, they disappear within 1 week of *in vitro* culture leaving a bed layer of phase-contrast gray cells (astrocytes and connective tissue cells) with ≤ 2 –5% phase-contrast dark, process-bearing cells (oligodendrocytes) on top (4–6). Thereafter, mature oligodendrocytes do not increase in number and rarely if ever incorporate [3 H]thymidine, suggesting that little or no cell division occurs (7). In

serum-supplemented medium these oligodendrocytes do not divide in response to several known mitogens: fibroblast growth factor, pituitary extract, myelin basic protein, epidermal growth factor, or insulin. However, autoradiographic and electron microscopic studies *in vivo* suggest that (i) the percentage of mature oligodendrocytes should be greater and (ii) oligodendroblasts, even myelinating oligodendrocytes in some cases, are capable of further proliferation postnatally (8–10) and in adult rodents after trauma (11).

Conventional culture methods appear to be suboptimal for the survival and proliferation of oligodendrocytes. Furthermore, the complexity, variability, and largely undefined nature of the biological fluid supplements preclude definition of the molecular growth requirements of oligodendrocytes. Thus, I have attempted to define the growth requirements of oligodendrocytes by using serum-free culture methods. The strategy of defining the requirements for growth of continuous cell lines expressing the phenotype of interest and applying this knowledge to culture of their normal cell counterparts has proven useful in the past for neurons and astrocytes (12–19). Although these cell lines are of tumor origin, they share some of the growth requirements of their normal cell counterparts. In addition, tumor cells often produce growth factors that act in an autocrine fashion to stimulate their own growth (20). After determining the growth requirements of the appropriate cell line, I then evaluate whether normal cell counterparts have any additional requirements.

I now describe the growth requirements for oligodendrocytes. I used the recently established CO-13-7 clonal cell line for the initial phase of this investigation. It is a somatic cell hybrid of a normal calf brain oligodendrocyte fused with a C6 glial tumor cell (21). Unlike the normal oligodendrocyte parent, the hybrid cells proliferate continuously as does the tumor cell parent. The C6 cell line expresses both astrocyte-specific (glial fibrillary acidic protein) and oligodendrocyte-specific [2',3'-cyclic nucleotide 3'-phosphodiesterase (EC 3.1.4.37), glycerol-3-phosphate dehydrogenase (EC 1.1.1.8), and the induction of glycerol-3-phosphate dehydrogenase by glucocorticoids] properties (22). In addition to the oligodendrocyte-specific markers present in C6 cells, CO-13-7 cells also contain galactocerebroside (GalCer; galactosylceramide), sulfatide [galactosyl(3-O-sulfate)-ceramide], and myelin basic protein (21). Thus, CO-13-7 cells exhibit a broad spectrum and substantial levels of expression of the six major biochemical properties of oligodendrocytes. The latter phase of this investigation used dissociated cultures of neonatal rat brain.

This study shows that sustained division of cell lines expressing oligodendrocyte properties and long-term survival of neonatal rat brain oligodendrocytes is possible using a chemically defined medium (CDM) and a permissive substratum. A significant increase in the percentage of mature

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: CDM, chemically defined medium; CNS, central nervous system; FCS, fetal calf serum; GalCer, galactocerebroside.

oligodendrocytes, a decrease in astrocytes, and no fibroblasts are obtained in O1 medium compared to serum-containing medium.

MATERIALS AND METHODS

Continuous Cell Lines. CO-13-7 and ROC-1 hybrid cell lines were obtained from F. A. McMorris (Wistar Institute of Anatomy and Biology, Philadelphia); 3T3 Swiss mouse cell line was provided by D. Carney of this institution. Stock cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (vol/vol) fetal calf serum (FCS); 5% (vol/vol) calf serum; 1.2 g of NaHCO_3 per liter; and 15 mM Hepes (pH 7.3). Medium was changed three times per week. Cells were grown in tissue culture flasks (Falcon; 25 cm^2) in 3–4 ml of medium in a humid atmosphere of 95% air/5% CO_2 at 37°C and were subcultured before confluence with 0.05% trypsin/0.5 mM EDTA in phosphate-buffered saline (PBS; pH 7.0) without Ca^{2+} or Mg^{2+} . After detachment, cells were enumerated with a Coulter counter.

Dissociated Neonatal Rat Brain Cultures. Sprague-Dawley rat littermates (1–3 days postnatal) were dipped in 70% ethanol and then decapitated. Whole brains were removed and collected in a dish containing Ham's F12 medium with 10% (vol/vol) FCS and antibiotics (50 μg of penicillin/ml, 50 μg of streptomycin/ml, 10 μg of neomycin/ml and 50 μg of gentamycin/ml). Pooled brains were minced with scissors, dissociated by sieving through metal and/or nylon meshes of successively smaller pore sizes (380 to 30 μm), centrifuged 1 min at low speed, and resuspended in the above medium without gentamycin; 5×10^6 cells/25 cm^2 were plated into culture vessels. After 36 hr, plating medium was exchanged for DMEM with 10% (vol/vol) calf serum and no antibiotics. The following day unattached and loosely attached cells were removed from original culture vessels, centrifuged 1 min at low speed, resuspended in unsupplemented DMEM, and transferred to culture vessels containing DMEM with 10% (vol/vol) calf serum or O1 medium (see below). Thereafter, tertiary cultures were obtained by detaching cells with trypsin/EDTA, adding an equal volume of 0.05% soybean trypsin inhibitor, centrifuging 1 min at low speed, resuspending the cell pellet in fresh medium, and replating as for secondary cultures. Cells were maintained at 37°C as above. Half the culture medium was exchanged for fresh medium every 3–5 days for secondary and tertiary cultures.

O1 Medium and Substratum. O1 medium consists of DMEM (with NaHCO_3 and Hepes as above) with 5 μg of bovine insulin/ml, 50 μg of human transferrin/ml, 30 nM sodium selenite, and 10 ng of biotin/ml. Concentrated stock solutions (500 $\times$) of supplements were prepared and stored as follows. Transferrin and biotin were dissolved in Hank's balanced salt solution (HBSS), aliquoted, and stored at -20°C . Sodium selenite was first dissolved in purified H_2O (0.5 mM), and a secondary stock solution was prepared in HBSS as above. Insulin was dissolved in 0.01 M HCl and stored at 4°C only for a maximum of 6 wks.

The culture substratum was modified with polylysine (2 $\mu\text{g}/\text{cm}^2$) and fibronectin (0.6–1.2 $\mu\text{g}/\text{cm}^2$) when cells were plated directly into O1 medium. Culture vessels were incubated with 0.05 mg of polylysine/ml for 5 min at room temperature, followed by a H_2O wash. Fibronectin was purified from human plasma (23) and stored in aliquots at 4°C only. It was added to O1 medium prior to the cell inoculum.

Indirect Immunofluorescence. Cells were fixed with 3.7% (wt/vol) paraformaldehyde in PBS (pH 7.4) containing 0.02% NaN_3 and were immunostained at room temperature according to the method of Norton *et al.* (24). After washing with PBS, background staining is reduced by incubating cells 15 min with solution A [10% (vol/vol) goat normal serum, 10% (vol/vol) calf serum, 40% (vol/vol) PBS, and 40% (vol/vol)

DMEM]. This is replaced with rabbit anti-GalCer (diluted 1:50 with solution A) for 30 min. After washing with PBS, goat anti-rabbit IgG coupled to rhodamine (diluted 1:50 with solution A) is added for 30 min. After washing again, cells are fixed with cold 5% (vol/vol) acetic acid/95% (vol/vol) EtOH for 10 min at -20°C . After extensive washing to regain pH 7.3, cells are mounted in 30% (vol/vol) glycerol in PBS, covered with a glass coverslip, and sealed with nail varnish. Cells are examined with a Nikon fluorescence microscope with rhodamine filters, epi-illumination, and phase contrast optics.

Materials. DMEM (430-2100), Ham's F12 medium (430-1700), penicillin/streptomycin/neomycin (600-5640), and gentamycin (600-5710) were from GIBCO; fetal calf and calf sera, HBSS (9222 and 9230), trypsin (9336), and EDTA (9314) were from Irvine Scientific; biotin was from Calbiochem-Behring; spectrographically pure sodium selenite was from Johnson Matthey (London); Hepes (H-3375), NaN_3 (S-2002), soybean trypsin inhibitor (T-9003-1S), poly(D-lysine) (P-7886; M_r 30,000–70,000), human transferrin (T-2242), bovine insulin (I-5500), and other materials were from Sigma. Rabbit anti-GalCer (24) was generously provided by K. Fields (Albert Einstein College of Medicine, New York), and mouse monoclonal anti-Thy 1.1 (25) by R. Pruss (National Institute of Mental Health, Bethesda, MD); mouse monoclonal anti-glial fibrillary acid protein was purchased from Labsystems (Chicago); and fluorescein- or rhodamine-labeled goat anti-rabbit or -mouse IgG was from Cappel Laboratories (Malvern, PA).

RESULTS

CO-13-7 Cell Culture. DMEM was selected as the basal medium since it contains a high concentration of glucose (4.5 g/liter) and other components. A dose-dependent decrease in newborn mouse brain oligodendrocytes, but not astrocytes, occurs with lower glucose levels (26). Growth curves of CO-13-7 cells in various media are shown in Fig. 1. Cell division does not continue in unsupplemented DMEM, whereas proliferation is logarithmic in serum-supplemented or O1 medium. The growth rate in O1 medium is about 70% of that in serum-containing medium. Long-term culture is possible using this CDM; CO-13-7 cells have been serially subcultured six times in O1 medium and been maintained for as long as 3 months.

FIG. 1. Growth curve for CO-13-7 cells. (■) Unsupplemented DMEM; (▲) O1 medium; (●) DMEM with 5% (vol/vol) FCS and 5% (vol/vol) calf serum. Cell inoculum was 100,000 cells per 8 cm^2 . Triplicate cultures were counted on the days indicated; SD varied <10%. Media were changed on day 5.

Deletion of insulin, transferrin, fibronectin, or polylysine results in a significant reduction in CO-13-7 cell number on day 4 (Table 1). The deficit resulting from deletion of sodium selenite or biotin is not apparent on day 4 but appears later. The stringency of these requirements is: polylysine substratum > insulin > fibronectin substratum = transferrin. As in my previous studies with other neural cell types (16, 19), the combined effect of all components is synergistic; each component alone has little or no effect and together the response is greater than the sum of individual effects (data not shown). The growth response to CO-13-7 cells as a function of supplement concentration is shown for insulin and transferrin in Fig. 2. The maximum number of cells is obtained with 1–5 μ g of insulin/ml and 50 μ g of transferrin/ml.

The following molecules had no growth-stimulating effect on CO-13-7 cells when added to O1 medium: 3,3',5-triiodo-L-thyronine, 0.5 nM to 5 μ M; epidermal growth factor, 0.01–100 ng/ml; neurotensin, 0.01–3 ng/ml; angiotensin II, 65 fg/ml to 6.5 ng/ml; cyanocobalamin (vitamin B12), 0.1–10 μ g/ml; choline chloride, 0.1–1 mM; carnitine hydrochloride, 1–100 μ M; AlCl_3 , 1–100 μ M; VOSO_4 , 1–10 μ M; ZnSO_4 , 1–30 μ M; CuSO_4 , 1–100 μ M; LiCl , KCl , or NaCl , 0.1–3 mM; and veratridine, 75 μ M. In contrast, several factors had growth-inhibiting effects: 2.5S nerve growth factor, 0.01–100 ng/ml; VOSO_4 , 30–100 μ M; and choline chloride, 3–30 mM.

Somatomedin [insulin-like growth factor (IGF) I and II] receptors are present in brain (27); and insulin has a low affinity for IGF I receptors. Thus, I tested whether multiplication stimulating activity, which binds to IGF II receptors, could cause a further increase in CO-13-7 cell number if added to a saturating dose of insulin. Multiplication stimulating activity (1–150 ng/ml) had no effect in the absence or presence of insulin.

I also plated CO-13-7 cells on cell-free extracellular matrices produced by the following cells grown in the indicated CDM: CO-13-7 (O1 medium), U-251 MGsp human astrocytoma (G2 medium; ref. 14), LA-N-1 human neuroblastoma (N3 medium; ref. 16), and neonatal rat brain astrocytes (G5 medium; ref. 18). Cells were removed at confluence with 0.02% EGTA in PBS without Ca^{2+} or Mg^{2+} , and any residual cells were hypotonically lysed. There was no difference in the number of CO-13-7 cells when cultured on any of these cell-free extracellular matrices compared to a polylysine and fibronectin-modified substratum.

Fig. 3 shows the morphology of CO-13-7 cells is similar in serum-supplemented or O1 medium; cells are phase-contrast dark and process bearing in both conditions but appear to be less mobile in O1 medium, forming flat clusters (Fig. 3C). Cells are in poor condition in unsupplemented DMEM (Fig. 3B).

I then evaluated the expression of several oligodendrocyte-specific properties of CO-13-7 cells maintained in O1 medium. The cells continue to express immunoreactive GalCer, and there is a 2-fold increase in 2',3'-cyclic nucleotide

FIG. 2. Dose-dependent growth of CO-13-7 cells for insulin (A) and transferrin (B). In each experiment, the concentration of all other O1 components was constant. Cell inoculum was 100,000 cells per 8 cm². Values are expressed as the mean $\pm$ SD of triplicate cultures counted on day 4.

3'-phosphodiesterase activity (28) compared to serum-supplemented cultures 4 days after replating: 2345 vs. 1127 nmol of 2'-adenosine monophosphate per min per mg of protein.

Finally, I tested the ability of O1 medium to sustain the division of several other cell lines, representing a variety of cell types (Table 2). The ROC-1 hybrid cell line was established by F. A. McMorris (Wistar Institute, Philadelphia) and differs from the CO-13-7 cell line in having a rat rather than calf oligodendrocyte parent. It expresses several oligodendrocyte-specific biochemical properties (F. A. McMorris, personal communication). Only ROC-1 cells continue to proliferate and can be subcultured in O1 medium. In contrast, glioma and fibroblast cells do not continue dividing, and neuroblastoma cells do not even survive. Thus, O1 medium has oligodendrocyte-specificity with regard to growth stimulation of these cell lines.

Neonatal Rat Brain Cell Culture. I then determined whether O1 medium would support growth of normal oligodendrocytes derived from neonatal rat brains. Representative fields of 24-day-old cultures are shown in Fig. 4 after two subcultures into serum-supplemented or O1 medium. Cells were immunostained for GalCer, the major myelin glycolipid and an early cell surface marker specific for mature oligodendrocytes (29). Fig. 4A shows a single cell in serum-supplemented medium is GalCer-positive, <2% of the total population shown in Fig. 4B. In contrast, Fig. 4C shows several GalCer-positive cells in O1 medium, one with very long, extensively branched processes characteristic of mature oligodendrocytes. Fig. 4E contains clusters of GalCer-positive cells in O1 medium, suggesting that proliferation has occurred. Specificity of the staining was indicated by sub-

FIG. 3. Phase-contrast micrographs of CO-13-7 cells grown in various media. Cells are shown 3 days after plating. DMEM was supplemented with 5% (vol/vol) FCS and 5% (vol/vol) calf serum (A), no addition (B), or O1 components (C). The substratum was modified for C by polylysine precoating and addition of fibronectin to O1 medium prior to the cell inoculum, which was 100,000 cells per 8 cm². (Bar = 100 μ m.)

Table 1. Growth response of CO-13-7 cells to single deletions of O1 components and substratum modifications

Deletion	Cells/8 cm² (× 10⁻⁵)	% control
None	4.10 $\pm$ 0.33	100 $\pm$ 8
Biotin	3.85 $\pm$ 0.25	94 $\pm$ 6
Sodium selenite	3.82 $\pm$ 0.62	93 $\pm$ 15
Transferrin	2.32 $\pm$ 0.24	57 $\pm$ 6
Fibronectin substratum	2.30 $\pm$ 0.24	56 $\pm$ 6
Insulin	1.62 $\pm$ 0.27	40 $\pm$ 7
Polylysine substratum	1.11 $\pm$ 0.13	30 $\pm$ 3

Cells were plated directly into O1 medium at a density of 100,000 cells per 8 cm² and counted on day 4. Values are expressed as the mean $\pm$ SD of triplicate cultures and also as a percentage of the control (no deletion).

Table 2. Cell-type specificity of O1 medium

Cell line	Origin	% control
ROC-1	Rat oligodendrocyte-C6 rat glioma	60 $\pm$ 3
C6-2B	Rat glioma	14 $\pm$ 1
3T3	Mouse fibroblast	12 $\pm$ 1
B104	Rat neuroblastoma	0 $\pm$ 0

Cells were plated directly into O1 medium at a density of 50,000 cells per 8 cm² (C6-2B, 3T3, B104) or 100,000 cells per 8 cm² (ROC-1) and counted on day 4. Values are expressed as a percentage of the control [DMEM with 5% (vol/vol) FCS and 5% (vol/vol) calf serum].

stituting normal rabbit serum for anti-GalCer; no significant staining was seen. Other cultures immunostained for glial fibrillary acidic protein, an astrocyte-specific marker (30), and Thy 1.1, a neuronal and fibroblast marker (5), confirm that astrocytes are the only other cell type present. The number of astrocytes is markedly lower in O1 medium. Random field counts indicate that 1–2% of cells in serum-supplemented medium are GalCer-positive oligodendrocytes, whereas in O1 medium 20–40% are GalCer-positive. There are about 15–20 times more oligodendrocytes in O1 than in serum-supplemented medium. Because of the high proportion of clustered oligodendrocytes, enumeration is difficult, and these figures may be underestimated.

More recently, I have plated primary cells directly into O1 medium on surfaces modified with polylysine and either fibronectin or laminin. Cells of typical oligodendrocyte morphology, with long, extensively branched processes (some ending in membranous expansions), survive well in these conditions. There appears to be little difference if laminin is substituted for fibronectin. Cultures were maintained in a healthy condition for longer than 2 months until terminated. In addition, primary cells maintained in serum-containing medium for 53 days (with two subcultures) and then subcultured directly into O1 medium have been maintained in it for an additional 7 months. These cultures contain fewer cells of oligodendrocyte morphology than cultures initially plated in

O1 medium, but they also exhibit elaborately branched processes.

DISCUSSION

Conventional culture methods are not ideal for growth of oligodendrocytes. In the present study I have identified the requirements for proliferation of cell lines expressing differentiated properties of oligodendrocytes and for long-term survival of mature oligodendrocytes derived from neonatal rat brains. I have replaced serum with purified soluble and substratum factors. Cultures in O1 medium contain a significantly higher percentage of mature oligodendrocytes, a decreased number of astrocytes, and no fibroblasts compared to serum-supplemented medium. Thus, in addition to not needing neurons for long-term survival, oligodendrocytes do not require fibroblasts either. My data suggest that the brain cultures may exhibit increased survival or proliferation of oligodendrocyte precursor cells and/or increased survival of more mature oligodendrocytes.

A number of *in vivo* and *in vitro* observations are consistent with the role of O1 medium supplements in the growth and development of oligodendrocytes. For example, CNS neural cells contain high levels of insulin and its receptors, and they are not regulated by peripheral levels of this hormone, suggesting local synthesis (31). The addition of 5 μ g of bovine insulin/ml to dissociated neonatal rat brain cells cultured in FCS-supplemented medium results in an increase in morphologically identified (but not quantified) oligodendrocytes (32). Transferrin is an iron-transport protein found in high concentrations in rat plasma and cerebrospinal fluid. It is also present in developing brain despite the blood-brain barrier, suggesting that it is also locally synthesized. Indeed, fetal brain and choroid plexus synthesize transferrin and several other plasma proteins (33). Moreover, the amount of rat brain transferrin mRNA, detected with a cDNA probe, increases postnatally to a maximum at 60 days (34). The trace element selenium reaches adult levels in rats by 2 weeks after birth (35). About one-fifth of it is present in glutathione peroxidase, which protects membranes by converting lipid peroxides to their corresponding monohydroxy-unsaturated fatty acids. The function of the remainder of the selenium, mostly associated with protein, was not determined. Ten-fold higher activities of glutathione peroxidase are found in isolated glia than neurons from adult rat brain (36). Finally, the vitamin biotin is the prosthetic group for several key enzymes in carbohydrate and lipid metabolism, but there are no studies relating it to neural cell function specifically.

Several other studies describe oligodendrocyte development in CDM after initial exposure to serum. Raff *et al.* (37) reported that glial precursor cells develop into Type 2 (fibrous) astrocytes in DMEM containing serum or into oligodendrocytes in a modified N2 medium (12). Albumin, thyroxine (T₄), and triiodothyronine (T₃) were added to N2 supplements (insulin, transferrin, selenium, progesterone, and putrescine). N2 medium was optimized for neuronal survival or neuroblastoma proliferation and is suboptimal for Schwann cells and astrocytes (13, 16, 17). Modified N2 medium favors oligodendrocyte differentiation in 7-day-old rat optic nerve cultures prepared from enzymatically dissociated tissue. In contrast, I find that the addition of progesterone, putrescine, albumin, T₄, or T₃ is not required in my culture system. A different CDM for culture of isolated oligodendrocytes from 5- to 10-day-old rat cerebra was described by Koper *et al.* (38). Cells were maintained for 30 days in this CDM, which is very complex and contains 19 supplements, including the 4 supplements in O1 medium. After my initial data were presented (39), a CDM similar to the one reported here was described by Eccleston and Silberberg (40). Trypsin-dissociated newborn rat brain cells

FIG. 4. Immunostaining for galactocerebroside (GalCer) in dissociated neonatal rat brain cultures. After 24 days (and 2 subcultures) cells were stained with rabbit anti-GalCer followed by goat anti-rabbit IgG coupled to rhodamine and viewed with fluorescence (A, C, and E) and phase-contrast (B, D, and F) optics. DMEM was supplemented with 5% (vol/vol) FCS and 5% (vol/vol) calf serum (A and B) or O1 components (C–F). A and B, C and D, and E and F represent corresponding fields. (Bar = 50 μ m.)

were cultured in DMEM/Ham's F12 medium (1:1; vol/vol) containing insulin, transferrin, selenium, and T₃. Although increased numbers of mature oligodendrocytes appeared in their cultures, cells began to detach from the culture surface after 3 weeks.

Thus, O1 medium has several advantages over the various CDM described above. It is able to support proliferation of cell lines expressing oligodendrocyte properties in addition to maintaining a significantly higher percentage of mature oligodendrocytes, fewer astrocytes, and no fibroblasts compared to serum-supplemented medium. Also, it has fewer components than the other CDM and is notably lacking thyroid hormone. Cells can be subcultured several times in O1 medium and can even be plated directly into it, without initial culture in serum-containing medium, if the surface is modified with polylysine and fibronectin. Furthermore, cultures have been maintained in a healthy condition for longer periods of time in O1 medium than in the other CDM, i.e., several months vs. weeks. In addition, I also find that O1 medium can maintain for long periods of time isolated adult oligodendrocytes from both bovine and rat brain (41), suggesting that O1 medium may not be species specific nor developmental stage specific.

Elucidation of thyroid hormone action in the CNS has been hindered by the heterogeneity of the tissue. The role of this hormone in oligodendrocyte proliferation and maturation *in vivo* and *in vitro* is reviewed by Legrand (42). Hypothyroid rats have normal numbers of oligodendrocytes, whereas hyperthyroid rats are deficient in oligodendrocytes. T₃ also has no effect on [³H]thymidine incorporation or DNA content of fetal rat brain reaggregate cultures (43). On the other hand, 2',3'-cyclic nucleotide 3'-phosphodiesterase activity and myelinogenesis are impaired in the hypothyroid state. Thus, thyroid hormone is not necessary for the proliferation of oligodendroblasts or the survival of mature oligodendrocytes. It also does not appear to be necessary for the expression of GalCer in O1 medium. Whether it is required for optimal levels of 2',3'-cyclic nucleotide 3'-phosphodiesterase or for myelinogenesis in CDM has not yet been determined. It is also not known to what extent thyroid hormone effects on oligodendrocytes are direct or mediated through astrocytes or neurons.

Knowledge of the growth requirements of oligodendrocytes makes possible the use of a chemically defined medium and substratum, which will greatly extend the range, reproducibility, and ease of interpretation of future experiments. This will benefit studies of the regulation of oligodendrocyte proliferation and differentiation.

I thank Pat Neal for expert technical assistance, Diane Pye and Sharon Bloom for their participation in the experiments, and Rita Schmidt for manuscript preparation. This research was supported by National Institutes of Health Grant NS 20375.

- Gilbert, P., Kettenmann, H. & Schachner, M. (1984) *J. Neurosci.* **4**, 561-569.
- Reynolds, R. & Herschkowitz, N. (1984) *Brain Res.* **322**, 17-31.
- Pfeiffer, S. (1984) in *Oligodendroglia*, ed. Norton, W. (Plenum, New York), pp. 233-298.
- Sensenbrenner, M. (1977) in *Cell, Tissue, and Organ Cultures in Neurobiology*, eds. Fedoroff, S. & Hertz, L. (Academic, New York), pp. 191-213.
- Raff, M., Fields, K., Hakomori, S., Mirsky, R., Pruss, R. & Winter, J. (1979) *Brain Res.* **174**, 283-308.
- Mirsky, R., Winter, J., Abney, E., Pruss, R., Gavrilovic, J. & Raff, M. (1980) *J. Cell Biol.* **84**, 483-494.
- Pruss, R., Bartlett, P., Gavrilovic, J., Lisak, R. & Rattray, S. (1981) *Brain Res.* **254**, 19-35.
- Skoff, R. (1980) *Path. Res. Pract.* **168**, 279-300.
- Sturrock, R. (1982) *Adv. Cell. Neurobiol.* **3**, 3-33.
- Wood, P. & Bunge, R. (1984) in *Oligodendroglia*, ed. Norton, W. (Plenum, New York), pp. 1-46.
- Ludwin, S. (1984) *Nature (London)* **308**, 274-275.
- Bottenstein, J. & Sato, G. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 514-517.
- Bottenstein, J., Skaper, S., Varon, S. & Sato, G. (1980) *Exp. Cell Res.* **125**, 183-190.
- Michler-Stuke, A. & Bottenstein, J. (1982) *J. Neurosci. Res.* **7**, 215-228.
- Michler-Stuke, A. & Bottenstein, J. (1982) *Cold Spring Harbor Conf. Cell Proliferation* **9**, 959-971.
- Bottenstein, J. (1983) *Adv. Cell. Neurobiol.* **4**, 333-379.
- Bottenstein, J. (1983) *Curr. Methods Cell. Neurobiol.* **4**, 107-130.
- Michler-Stuke, A., Wolff, J. & Bottenstein, J. (1984) *Int. J. Dev. Neurosci.* **2**, 575-584.
- Bottenstein, J. (1985) in *Cell Culture in the Neurosciences*, eds. Bottenstein, J. & Sato, G. (Plenum, New York), pp. 3-43.
- Todaro, G. (1982) *Natl. Cancer Inst. Monogr.* **60**, 139-147.
- McMorris, F. A., Miller, S., Pleasure, D. & Abramsky, O. (1981) *Exp. Cell Res.* **133**, 395-404.
- Pfeiffer, S. (1977) in *Cell, Tissue, and Organ Cultures in Neurobiology*, eds. Fedoroff, S. & Hertz, L. (Academic, New York), pp. 287-346.
- Bottenstein, J. & Sato, G. (1980) *Exp. Cell Res.* **129**, 361-366.
- Norton, W., Farooq, M., Fields, K. & Raine, C. (1983) *Brain Res.* **270**, 295-310.
- Lake, P., Clark, E., Khorshidi, M. & Sunshine, G. (1979) *Eur. J. Immunol.* **9**, 875-886.
- Zuppinger, K., Wiesmann, U., Siegrist, H., Schafer, T., Sandru, L., Schwarz, H. & Herschkowitz, H. (1981) *Pediatr. Res.* **15**, 319-325.
- Sara, V., Hall, K., Rodeck, C. & Wetterberg, L. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 3175-3179.
- Prohaska, J., Clark, D. & Wells, W. (1973) *Anal. Biochem.* **56**, 275-282.
- Raff, M., Mirsky, R., Fields, K., Lisak, R., Dorfman, S., Silberberg, D., Gregson, N., Leibowitz, S. & Kennedy, M. (1978) *Nature (London)* **274**, 813-816.
- Bignami, A., Eng, L., Dahl, D. & Uyeda, C. (1972) *Brain Res.* **43**, 429-435.
- Havrankova, J., Brownstein, M. & Roth, J. (1981) *Diabetologia* **20**, 268-273.
- McMorris, F. A. (1983) *J. Neurochem.* **41**, 506-515.
- Dziegielewska, K., Evans, C., New, H., Reynolds, M. & Saunders, N. (1984) *Int. J. Dev. Neurosci.* **2**, 215-222.
- Levin, J., Tuil, D., Uzan, G., Dreyfus, J. & Kahn, A. (1984) *Biochem. Biophys. Res. Commun.* **122**, 212-217.
- Prohaska, J. & Ganther, H. (1976) *J. Neurochem.* **27**, 1379-1387.
- Savolainen, H. (1978) *Res. Commun. Chem. Pathol. Pharmacol.* **21**, 173-176.
- Raff, M., Miller, R. & Noble, M. (1983) *Nature (London)* **303**, 390-396.
- Koper, J., Lopes-Cardozo, M., Romijn, H. & van Golde, L. (1984) *J. Neurosci. Methods* **10**, 157-169.
- Bottenstein, J. (1985) in *Growth and Differentiation of Cells in Defined Environments*, eds. Murakami, H., Yamane, I., Barnes, D., Mather, J., Hayashi, I. & Sato, G. (Springer, Tokyo), pp. 25-30.
- Eccleston, A. & Silberberg, D. (1984) *Dev. Brain Res.* **16**, 1-9.
- Norton, W., Farooq, M. & Bottenstein, J. (1986) in *Dynamic Properties of Glial Cells: Cellular and Molecular Aspects*, eds. Grisar, T., Franck, G., Hertz, L., Norton, W., Sensenbrenner, M. & Woodbury, D. (Pergamon, Oxford), in press.
- Legrand, J. (1984) in *Neurobehavioral Teratology*, ed. Yanai, J. (Elsevier, New York), pp. 331-363.
- Honegger, P. & Lenoir, D. (1980) *Brain Res.* **199**, 425-434.